Muramyl dipeptide responsive pathways in Crohn's disease: from NOD2 and beyond

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Muramyl dipeptide responsive pathways in Crohn's disease: From NOD2 and beyond

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Abstract

Crohn's disease (CD) is one of main disease entities under the umbrella term chronic inflammatory bowel disease. The etiology of CD involves alterations in genetic, microbiological, and immunological factors. This review is devoted to the role of the bacterial wall compound muramyl dipeptide (MDP) for the activation of inflammatory pathways involved in the pathogenesis of CD. The importance of this molecule is underscored by the fact that [1)] MDP, which is found in most Gram-negative and -positive bacteria, is able to trigger several immunological responses in the intestinal system, and [2)] that alterations in several mediators of the MDP response including – but not restricted to – nucleotide oligomerization domain 2 (NOD2) are associated with CD. The normalization of MDP signaling is one of several important factors that influence the intestinal inflammatory response, a fact which emphasizes the pathogenic importance of MDP signaling for the pathogenesis of CD. The important aspects of NOD2 and non-NOD2 mediated effects of MDP for the development of CD are highlighted, as well as how alterations in these pathways might translate into the development of new therapeutic strategies.

Keywords

ATG16L1 · autophagy · CD · IBD · innate lymfoid cells · inflammasome · MDP · NF-κB · nucleotide oligomerization domain 2 · Paneth cells · peptidoglycan · Toll-like receptor

Introduction

The innate immune system is the first line of defense in the intestinal tract, and it possesses a unique feature in recognizing microbial components. The pathogens are detected by pattern recognition receptors (PRRs), which recognize a wide array of conserved structures of microorganisms, called the pathogen-associated molecular patterns (PAMPs). The PRRs sense specific viral and bacterial structures, and subsequently initiate various immunological responses. PRRs are located on either the membrane surface, *e.g.* Toll-like receptors (TLRs), or inside the cytoplasm, *e.g.* NOD-like receptors (NLRs) [1].

Muramyl dipeptide (MDP) is an immunoreactive peptide found in the peptidoglycan (PGN) motif which encodes "the building blocks" of bacterial cell walls [2]. MDP can activate several immunological signaling pathways, including the nucleotide oligomerization domain 2 (NOD2, a NLR member) dependent pathway via specific interaction between NOD2 and MDP; resulting in activation of nuclear factor-κB (NF-κB), a ubiquitous transcription factor which induces expression of pro-inflammatory cytokines [2]. Multiple studies have linked NOD2 to several severe immunological dysfunctions such as graft-versus-host disease [3], enhanced mortality during sepsis [4, 5] , and Crohn's disease (CD) [6, 7], indicating that MDP might play a pathophysiological role in these disorders. The *NOD2* gene, which initially was identified as *NOD1*-related gene, was found to have a highly restricted expression in monocytes [8]. The major effects of *NOD2* in the pathogenesis of CD were later revealed by Hugot *et al.[6]* and Ogura *et al.*[7], who identified three disease linked polymorphisms in the *NOD2* gene. As described below, MDP also affects other PRRs than NOD2.

CD is one of the two a main entities of inflammatory bowel disease (IBD), potentially affecting any part of the gastrointestinal tract, however, most commonly the terminal ileum or the perianal region [9, 10]. The etiology of IBD is still unknown, but considering epidemiological, genetic and immunological data together, IBD has a multifactorial etiology in which genetic alternations and environmental factors (i.e. microbial behavior) interact to produce the immunological background of the disease [11]. Accordingly, it is becoming increasingly evident that an impaired innate immunity plays a crucial role in CD [12].

In this review, the recent studies on MDP-mediated pathways in the innate immunity of CD are summarized covering both NOD2 dependent and NOD2 independent pathways. As described in detail below, MDP signaling is impaired on different levels in CD, and animal studies suggest that reversal of these pathways reduces intestinal inflammation. Revealing such mechanisms might lead to identification of new potential therapeutic approaches of this devastating disorder.

MDP intracellular delivery

In most Gram-negative and -positive bacteria the PGN represents a key component of the cell wall with an important impact on maintaining the structural integrity of plasma membranes and anchoring cell components such as lipoproteins [2]. The pivotal role of PGN in the bacterial pathogenesis is clearly highlighted by the fact, that the most frequently used antibiotics are targeting the PGN biosynthesis pathway [13]. MDP is a cleavage product and the smallest bioactive motif of PGN that specifically binds to NOD2 and it leads to an activation of NOD2-mediated signal pathways and other pathways [14]. In general, the structure of PGN contains a repetition of disaccharides (glycans) cross-linked by short chains of amino acids (peptides) to form a lattice surrounding the entire cell. MDP consists of N-acetylmuramic acid linked to the two amino acids: D-Ala and D-isoGln (or D-Glu) [15]. The structure of MDP is

unique in the way that MDP analogues composed of other amino acid leads to a reduced or inhibited ability to induce a biological response, also called "*the priming effect*" [2, 16].

There is accumulating evidence suggesting that NOD2 might be a selective bacterial sensor due to the fact that *N*-acetyl MDP, which is found in most bacteria, less efficiently activates NOD2 than *N*-glycolyl MDP – which is found in *mycobacteria* and related *Actinomycetes* species [17]. This is of interest since CD has been associated with *mycobacteria* infections [18]. Accordingly, Coulombe et al. showed, that *N*-glycolyl MDP was more efficacious than *N*-acetyl MDP at inducing ovalbumin-specific T cell immunity in a model of adjuvancy. Further, *N*-glycolyl MDP has been found to be more potent than *N*-acetyl MDP in activating NOD2-mediated RIP2 polyubiquitination, NF-κB, as well as c-Jun N-terminal kinase (JNK) [17]. A recent *in vivo* experiment has supported this finding in a setting where intravenously administrated MDP has shown protective antiviral activity in mice exposed for influenza A virus (IAV). In this model, *N*-glycolyl MDP showed more potency than *N*-acetyl MDP and with a greater antiviral activity against IAV [19].

In both Gram-negative and -positive bacteria, the PGN has to be cleaved during cell growth by autolysis which allows attaching/inserting of new material into the existing cell wall. The loss of PGN resulting from this cleavage is termed "*PGN turnover*". However, these turnover products are normally captured and reutilized by the cell in another process named "*PGN recycling*", which is typically observed in studies with the Gram-negative bacteria, e.g. *Escherichia coli* [20]. Whether PGN is also recycled in Gram-positive bacteria is basically unknown.

A number of studies have focused on identifying the mechanisms by which the extracellular NOD2 ligands reach the cytosolic receptor and trigger downstream effector functions (Fig.1). The extracellular MDP can get access to the host cytosol through several ways. First, MDP can be taken up by the cell via membrane protein transporters, hPepT1 [21] and pannexin-1 [22]. Inhibition of these transporters leads to a downregulation of MDP localization from the extracellular space to the cytosol [22, 23]. Although hPepT1 is expressed in monocytes and human enterocyte cell lines like HT-29 cells, most protocols for stimulating primary cells with MDP relies on artificial permeabilisation of the cell membranes with liposome forming reagents like lipofectamine, suggesting that the hPepT1 system might be of limited efficiency in human cells [24]. Second, MDP similar to lipopolysaccharide (LPS) may use a clathrin- and dynamin-dependent endocytic pathway to cross the host cell membrane [25-27]. Clathrin and dynamin mediate a vesicle formation at the cell membrane, and an inhibition of clathrin or dynamin also leads to attenuated MDP-mediated signaling pathways [25]. The mechanism by which MDP is targeted to clathrin-coated pits for endocytosis remains unclear. Typically, endocytosis is thought to rely on adaptor transmembrane proteins, such as adaptor protein 2 (AP2), to mediate the recruiting process to coated pits by interaction to clathrin adaptors [28]. Third, it has been shown that phagocytic cells, interacting with the epithelium breaching microorganisms and microbial components, presents a key mechanism in allowing components of microorganisms to be exposed to intracellular receptors: Macrophages may generate such microbial components by ingesting whole bacteria and subsequently digesting them in the phagolysosomes [29, 30]. Thus, Herskovits *et al.* [29] have shown that NOD2 is activated in stimulated macrophages by bacterial ligands generated in the phagosome and transported to the cytosol [29]. Finally, PGN containing MDP can be delivered into the cytosol by the previously mentioned intracellular PGN turnover after internalization of invasive bacteria into the cells [31].

MDP-NOD2-mediated pathway

The NOD2 protein is located in the cytosol and is broadly expressed in macrophages, dendritic cells, and to a lower degree in intestinal epithelial cells (IECs), including Paneth cells of the small bowel [32]. The structure of NOD2 consists of two N-terminal caspase recruitment domains (CARDs), a centrally located nucleotide-binding domain (NBD), a C-terminal of site of ligand binding (e.g. MDP), and a leucine-rich repeat region (LRR) (Fig. 2) [8]. MDP binds to the LRR of NOD2, which subsequently activates NF-κB and mitogen-activated protein kinases (MAPKs) [33]. When activated, NF-κB translocates into the nucleus to initiate cell specific genetic programs, such as immune activation, inflammation, and cell development [34, 35]. Recent studies showed that the intestinal MDP-NOD2 pathway is tightly controlled by epithelium-associated lymphocytes, which specifically cleave the NOD2-detected MDP structure [36, 37]. Pro-inflammatory mediators produced by T helper 1 (Th1) cells, i.e. interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), upregulate the expression of NOD2 in intestinal epithelial cells [38]. This reflects the importance of NOD2 in the maintenance of the intestinal mucosal homeostasis, which actually links the innate and adaptive immunity.

The specificity of interaction between MDP and NOD2 is not completely understood. However, a recent study found that MDP binds directly to NOD2 with high affinity at a pH range of 5.0 to 6.5, compared with a pH range of 7.0 to 7.5 [39] which is the estimation of human cytosolic pH [40]. pH-dependent affinity of MDP-NOD2 interaction lines with earlier observations suggested that the internalization of MDP is optimal in the pH range from 5.5 to 6.5 [26]. MDP binding onto NOD2 causes a conformational change allowing a CARD-CARD interaction between NOD2 and RIP2 (receptor-interacting protein 2), a CARD-containing serine-threonine [41, 42]. The activation of RIP2 results in polyubiquitination of NEMO (NF-κB essential modifier), also named IKKγ (inhibitor of IκB kinase γ), which is found in complex with IKKα and IKKβ [43, 44]. IKKγ polyubiquitylation leads to phosphorylation of IKKβ, which in turn phosphorylates IκB (inhibitor of NF-κB) resulting in dissociation of IκB from NF-κB. Subsequently NF-κB can translocate into the nucleus where it promotes transcription of several pro-inflammatory and anti-inflammatory cytokines, such as interleukin-1 (IL-1), IL-2, IL-6, IL-8, IL-12, and TNF-α [32, 33, 45, 46] (Fig. 3).

NF-κB can additionally be activated by another pathway, known as "*the alternative pathway*", which has been classified according to the complex structure of NF-κB [35, 47]. The alternative pathway is, however, activated with much slower kinetics than the classical pathway [48]: The alternative NF-κB pathway is linked to the adaptive immune system, and it is involved in the development of lymphoid organs by regulating the generation of B and T lymphocytes [35]. The alternative pathway is activated by a small number of stimuli, including lymphotoxins (α and β), B cell activating factor (BAFF), receptor activator of nuclear factor kappa-B ligand (RANKL) as well as NOD2 [35, 48, 49]. The alternative pathway induces NF-κB inducing kinase (NIK) instead of NEMO to phosphorylate and activate the IKK complex, which facilitates induction of NF-κB dimers [34, 35]. The alternative pathway is a prerequisite for the development of secondary lymphoid tissues. Thus, mice with defects in the alternative pathway signaling are often associated with absence of lymph nodes, and a lack of specific lymphoid organization in spleen and thymus [50].

In IBD research, three independent gene variants of *NOD2* exist, a frame-shift mutation (c3020insC (SNP13)) and two missense mutations (R702W (SNP8); G908R (SNP12)). Further, some rare variants have been associated with

susceptibility to CD [6]. The frame-shift mutation results in an incompetent truncated NOD2 protein [7]. The *NOD2* gene variants cause a reduced response to MDP by generating a loss-of-function phenotype [51]. Thus, PBMCs from CD patients with the frame-shift mutation (c3020insC) express reduced levels of pro-inflammatory cytokines, e.g. TNF, IL-6 and IL-8 in response to MDP [15, 52, 53].

In addition to activation of the NF-κB-mediated pathway, NOD2 stimulation further results in the activation of the MAPKs: p38, JNK, and extracellular signal-regulated kinase (ERK); which are intracellular serine/threonine specific kinases involved in activation of multiple cellular processes, including cell growth, proliferation, differentiation, migration, inflammation and survival [54-58].

The MDP-NOD2 pathway and T helper cell regulation

During the last decade a number of hypotheses have discussed the regulatory immunological role of the *NOD2*-mutations in skewing Th differentiation. One of these hypotheses claims, that MDP NOD2-activation results in a down-modulation of TLR2-mediated Th1 responses, in which antigen-presenting cells (APCs) from *Nod2*-deficient mice exert increased amount of IL-12 supporting Th1 inflammatory responses [59]. This hypothesis is supported by observations that administration of MDP protects mice from acute experimental colitis [60, 61]. A second hypothesis suggests that defects in NOD2 signaling polarize the adoptive immune system towards the Th1/Th17-type, resulting in excessive Th1/Th17 cell-mediated inflammation, in which Th1 and Th17 cells are enriched at the inflamed gut together with increased levels of pro-inflammatory cytokines [62, 63]. In addition to the previous hypotheses, other studies indicate that activation of NOD2 signaling enhances the Th17 polarization. Thus, stimulation of human dendritic cells (DCs) with MDP has been shown to enhance NOD2-mediated production of IL-1β and IL-23 which in turn promotes IL-17 production by memory Th17 cells [64]. Moreover, a recently study reported by Geddes *et al*. [65] suggests a specific role for NOD2 in the early innate response. The authors used a mouse model in identifying an early NOD2-dependent Th17 response restricted to the acute phase of infections after treatment with pathogens, including *Salmonella Typhimurium* and *Citrobacter rodentium*. NOD2 expression by myeloid and somatic cells was a crucial factor in recognition of pathogens and thereby for a functional early Th17 response [65]. Further, impaired acute Th17 responses in *Nod2* knockout mice were associated with unaffected colonic colonization and diminished colonic inflammation at early stages, which is in contrast to an enhanced tissue destruction and increased bacterial translocation later in the course of the disease, indicating a protective role of the acute Th17 response in infection control [65]. However, treatment with human anti-IL-17A monoclonal antibodies has been revealed to be without any clinical effects in CD [66].

NOD2 seems also to be involved in immune regulatory pathways for maintaining the Th1/Th2/Th17 immune balance. For instance, mutations of NOD2 lead to an inhibited IL-10 expression, which is assumed to suppress Th1 cells [53, 67]. *NOD2* mutations suppress transcription of human *IL-10* by inhibitory activity of the nuclear ribonucleoprotein hnRNP-A1; a nucleocytoplasmic shuttling heterogeneous nuclear ribonucleoprotein that accompanies eukaryotic mRNAs from the active site of transcription to that of translation [67]. Further, human peripheral blood mononuclear cells (PBMCs) with the 3020insC frame shift *NOD2* mutation have an impaired production of the anti-inflammatory cytokine, IL-10 [53, 67]. Based on the observation that *IL-10* knockout mice develop colitis, and that loss of function mutations in the IL-10 receptor gene leads to early onset of CD in humans, this finding adds another aspect to the

importance of NOD2 for balancing various immune responses [68, 69]. Another hypothesis is based on observations where NOD2 is found to have a reciprocal interaction with MAPK-kinase transforming growth factor (TGF)-β-activated kinase 1; TAK1 [70]. NOD2 inhibits TAK1-induced activation of NF-κB [70], and *TAK1* gene silencing decreases the frequency of Th1 and Th17 cells [71]. This indicates that a loss-of-function phenotype of NOD2-mutations can lead to an increased TAK1-mediated Th17 cell activation. Recently, short non-coding RNAs, known as microRNAs (miRNAs), were profiled in DCs from patients with CD-associated mutations of *NOD2*, and a down-regulation in two miRNA clusters was revealed. Interestingly, these clusters were suggested to regulate a critical component of the Th1/ Th17 immune response [72].

The dysregulation of the immune balance in Th1/Th2/Th17 is further considered to be of importance for a defective Th2 immune response. Magalhaes et al. [73] showed that MDP activation of the NOD2-mediated pathway in DCs is insufficient to initiate Th2 immunity in absence of stromal-derived mediators, such as thymic stromal lymphopoientin (TSLP). TSLP is thought to promote Th2 immunity by an up-regulation of the expression of OX40 ligand (OX40L), which is a transmembrane protein expressed by APCs to recognize OX40, a receptor expressed by T cells [74, 75]. Magalhaes et al. found that NOD2 stimulation induced the up-regulation of OX40L expression on DCs. Moreover, *OX40* deficient mice had diminished capabilities in generating NOD2-drived Th2 immunity [73].

T-regulatory $Foxp3^+$ cells are important for the homeostasis of the intestinal immune response, and a lack of T regulatory cells has been associated with development of experimental colitis [76]. The dynamic of T-regulatory $Foxp3^+$ cells in IBD is still incompletely understood. Several studies have revealed an increased number of mucosal T regulatory cells in active CD [77, 78]. On the other hand, the number of T-regulatory $Foxp3^+$ cells has been found to be increased in the colonic lamina propria of children with CD treated with anti-TNF-α compared with both CD receiving conventional therapy and non-IBD controls patients with active CD [79]. Moreover, the number of T-regulator cells has also been reported to be higher in inflamed IBD mucosa than the peripheral blood [80]. It is therefore of interest, that the MDP-NOD2 pathway seems to be necessary for survival signaling in T-regulatory $Foxp3^+$ cells [81]. Further, T-regulatory $Foxp3^+$ cells from CD patients with disease associated gene variants of NOD2 were more susceptible to cell death than *wild type* NOD2 T-regulatory $Foxp3^+$ cells, and the number of T-regulatory $Foxp3^+$ cells were substantially decreased in NOD2 mutated patients [81]. The MDP-NOD2 pathway might therefore be crucial for the mucosal immune homeostasis as well.

MDP has been used as vaccine adjuvant, suggesting that MDP additionally stimulates B-cell adaptive immunity [82]. Taken together, these observations reflect the impact of the NOD2 signaling pathway in the dysregulation of adaptive responses related to the CD pathogenesis, although further clarification of the pathways involved is needed.

The MDP-NOD2 pathway in mucosal immunity

In the mucosal immunity, IECs provide a physical barrier with elemental signal-transduction functions in the maintenance of gut homeostasis. IECs consists of a number of epithelial cell types including Paneth cells [83]. The loss-of-function phenotype of *NOD2*-mutations has been linked to deficiencies of the epithelial-barrier function, which promotes the intestinal bacterial flora invasion and inflammation into the intestinal walls [84]. In Paneth cells, the expression levels of NOD2 increases in response to the intestinal bacteria [85, 86]. MDP stimulation of Paneth cells

leads to a NF-κB dependent expression and secretion of α-defensins, *i.e.* anti-microbial peptides, into the intestinal lumen [87-90]. Thus, α-defensins levels in CD patients with *NOD2* mutations are markedly reduced [91]. This reduction of α-defensins is consistent with activated intracytoplasmic digestion (i.e. crinophagy) of the secretory granules in CD Paneth cells [92]. In addition, *Nod2* knockout mice have been reported to possess a reduced mRNA expression of Paneth cell-derived α-defensins. These mice have an impaired intestinal mucosal homeostasis reflected in a reduced intestinal bacterial clearance, and an increased bacterial load of the terminal ileum [93, 94].

In addition, an abnormal MDP-NOD2 pathway has been associated with intestinal disorders and higher bacterial translocation. Thus, MDP stimulation of PBMCs from healthy individuals results in a 2-3-fold enhancement of TLR9 agonist stimulation of PBMC production of TNF-α and IL-8, whereas such an enhancement is not seen in PBMCs from CD patients bearing *NOD2* mutations [95], suggesting that the synergistic cytokine response between NOD2 and TLR9 might have a role in maintaining intestinal homeostasis. Moreover, it was recently found that MDP stimulation of murine colorectal epithelial cell lines leads to enhanced TLR2 response in the shape of significantly increased expression levels of chemokines, cytokines and tight junction molecules, e.g. claudin-3 and claudin-4 which are typically expressed from IECs in the colon to improve the barrier function [96]. Further, the *NOD2* genotype has been evidenced to influence the bacterial translocation in CD patients due to the fact, that the intestinal endotoxin accumulation in heterozygous SNP8 and SNP13 is stronger than in homozygos SNP12 patients and WT patients [97]. In addition, *Nod2* knockout mice are characterized by significantly higher tissue-associated intestinal bacterial levels [93, 94, 98]. *Nod2* knockout mice suffered from an impaired bacterial clearance after exposing with pathogens, e.g. *Listeria monocytogenes, Citrobacter rodentium, Salomonella and Mycobacterium tuberculosis* [99-102]. The increased bacterial colonization indicates a crucial role for NOD2 in the bacterial clearance. In a recent study, inhibition of NOD2 using carbamoyl phosphate synthetase/aspartate transcarbamylase/dihydroorotase (CAD) (a newly discovered NOD2-interacting protein), leads to a dramatic decrease in the clearance of *Salmonella* [103]. Thus, NOD2 mutations may play an essential role in the maintenance of intestinal homeostasis, and the results indicate that an impaired NOD2-mediated bacterial clearance might lead to accumulation of mucosal microbiota and secretion of higher levels of pro-inflammatory cytokines.

In mucosal immunity, innate lymphoid cell (ILC) populations, which are newly defined innate immune cells, have been shown to regulate the epithelial cell responses and maintaining intestinal homeostasis [104]. ILCs constitute of both well-established and recently identified T-cell populations and can be grouped into three major groups depending on specific transcription factors for their development and function: Group 1 ILCs depends on expression of T-bet (T-box expressed in T cells); Group 2 ILCs relay typically on expression of GATA3 (T-cell differentiation is the transcription factor GATA-binding protein 3); and Group 3 ILCs which require the expression of RORγt (retinoid-related orphan receptor γ) [104, 105]. Although only few studies have investigated the MDP signaling in ILCs, the presence of this pathway might be involved in the developmental and functional characteristics of ILCs. For instance Natural Killer (NK) cells, which belong to Group 1 ILCs and attend in the gut homeostasis by responding to commensally enteric bacteria through the innate immune system and cytolytic activity, express high levels of NOD2 [106], and MDP treatment of these cells leads directly to cell activation through the NOD2-mediated pathway [107]. In addition, MDP can also be sensed by TLR2 (described below) [108, 109] which is expressed on RORγt$^+$ ILCs [110]. Therefore, MDP might have an indirectly influence on ILCs through modulation of other cellular components of the

innate immunity. As previously mentioned, DCs stimulated with MDP express high levels of IL-23 and IL-1β [64], cytokines that can induce production of IL-22 from RORγt$^+$ ILCs [111, 112]. IL-22 has been reported to ameliorate intestinal inflammation in a murine model of experimental colitis [113], and bolster intestinal epithelial barrier function by upregulation of antimicrobial peptides [114]. Overall, additional studies are, however, required to clarify the MDP signaling and its influence on ILCs responses.

The MDP-NOD2 pathway and autophagy

The MDP-NOD2 signaling pathway might be a potential regulatory target for other upstream and downstream genes. An interesting illustration of such genes is the autophagy-related protein, 16-like 1 (ATG16L1), which has a key role in the process of cellular protein turnover as well as in handling the intracellular bacteria [90, 115]. The prevalence of *ATG16L1* polymorphism is high, as it exists in around 50% of the European population; however, the loss-of-function mutations in *ATG16L1* are associated to CD in form of a two-fold increased susceptibility [116-118]. Moreover, NOD2 is linked to ATG16L1 in the autophagy pathway, and is of importance for the recruitment of ATG16L1 at the site of bacterial entry [119]. The link between NOD2 and ATG16L1 was shown by LPS and MDP stimulation of GFP-LC3–transduced bone marrow–derived macrophages (BMDMs), a cell population expressing functional *Nod2*: The MDP-treated, but not LPS-treated, BMDMs showed an activated autophagy in *wild-type* macrophages, whereas macrophages isolated from *Nod2*-deficent mice did not activate this pathway [119].

Taken together these observations indicate that the MPD-NOD2 signaling pathway induces the innate immunity at multiple levels, thereby linking important aspects of the pathogenesis of CD to disturbances of the innate immune system. Therefore, the normalization of this signaling pathway may normalize the intestinal immunological homeostasis in subsets of CD patients with an impaired MDP signaling.

Cross-talk between NOD2 and other innate pathways

MDP sensing is not NOD2-restricted. Beyond the ability to activate NOD2-mediated signaling pathways, MDP can interact and signal through TLR2 [108, 109] . TLR2 recognizes MDP and activates NEMO-NF-κB pathway through two independently upstream signaling pathways [120]. Further, TLR2 triggers the association with myeloid differentiation primary-response protein 88 (MyD88), which subsequently can signal either through tumor-necrosis-factor-receptor-associated factor 6 (TRAF6), or by binding to receptor-interacting serine/threonine kinase (RICK) activating pathways similar to those involved in NOD2-signaling [42, 121-123]. Activation of TLR2 also facilitates induction of MAPKs signaling pathways [120, 124-126] (Fig. 4). *In vitro* data on MDP stimulation of splenic macrophages isolated from *Nod2*-deficient mice has shown an excessive NF-κB dependent IL-12 production, pointing to a possible NOD2-negative-regulatory role of the TLR2-mediated Th1 response, whereas the expression levels of other inflammatory cytokines produced by TLR2-signaling, such as TNF-α and IL-10, were unaffected [59]. In contrast, MDP stimulation of NOD2 and TLRs, including TLR2 in human PBMCs, leads to a positive synergistic effect, while MDP stimulation of mutant *NOD2* cells shows a lack of this immunological response, which results in an impaired NF-κB signaling pathway [52, 95]. This immunological response is, however, detrimental to the suggested pathogenesis of CD with elevated levels of NF-κB activation-dependent Th1 cytokines [127-129]. However, the effect of NOD2-activation on TLR2-mediated cytokine response has in another study been found to depend on the MDP activation dose

and the NOD2-genotype: Stimulation of *NOD2*-mutant monocytes with low doses of MDP results in a significant increase in the TNF-α production, as opposed to a down-regulated response at higher MDP doses. However, when monocytes isolated from *NOD2*-deficient patients are stimulated with high-dose MDP, divergent dynamics are revealed as the response of down-regulation lacks [130].

In addition to the previous mentioned NOD2-interaction, it has further been reported that NOD2 plays a major role in activation of innate immune antiviral responses. Sabbah et al. [131] reported that NOD2 is involved in induction of antiviral cytokines, including IFN-β, in response to external viral stimuli such as single-stranded RNA (ssRNA) and respiratory syncytial virus (RSV). The authors found that the activation mechanism requires NBD and LRR domain of NOD2 interaction with the mitochondrial antiviral signaling (MAVS) protein. The NOD2-MAVS protein complex facilitates the NOD2-mediated response by activating interferon-regulatory factor 3 (IRF3), which subsequently translocates to the nucleus and activates production of cytokines such as IFNs [132]. Additionally, recent studies have revealed a synergistic proinflammatory cytokine response in human PBMCs stimulated with RSV followed by MDP stimulation. Interestingly, this synergic response is absent in PBMCs isolated from CD patients homozygous for the 3020insC mutation in the *NOD2* gene [133]. These results suggest a critical role of NOD2 in this synergy and add an interesting aspect in how the *loss of function* mutations in *NOD2* can eliminate essential immune responses.

The inflammasome-mediated pathway

Another outcome of MDP recognizing by PRRs is the formation of inflammasomes, i.e. multiprotein complexes, which are involved in the conversion of pro-caspase-1 to casapase-1 through a CARD-CARD interaction. Active caspase 1 leads to cleavage of pro-inflammatory cytokines, such as pro-IL-1β and pro-IL-18, allowing more secretion of mature IL-1β and IL-18 [134, 135] (Fig. 5). Recently, Meinzer et al.[136] showed that *Yersinia pseudotuberculosis* induces an intestinal barrier dysfunction by subverting signaling of Nod2. They reported that acetylation of RICK and TAK1 kinases resulted in a reduced affinity for Nod2. Thus the free Nod2 interacts with and activates caspase-1 resulting in increased levels of IL-1β, which contributes to the induction of intestinal barrier dysfunction in Peyer's patches [136].

The NLR family encodes a key component in the innate immunity by microbe recognizing, and the activation of response signaling pathways. One of these pivotal pathways is the NLR control of inflammatory caspases, such as caspase-1 via formation of inflammasomes [137-139]. Four inflammasomes have been identified and named after the PRR that regulates their activity: NLRP1, NLRP3, NLRC4 and AIM2 (absent in melanoma 2), an intracellular DNA receptor [137, 140]. Three of them include PRRs from the NLR family: NLRP1, NLRP3 and NLRC4, which are composed of a C-terminal leucine-rich repeat domain, a central nucleotide-binding domain, and of an N-terminal effector domain, CARD or pyrin domain (PYD) (Fig. 5) [141]. NLRPs contain PYD, while CARD mediates downstream protein-protein interaction in NLRCs [142]. Recently, AIM2, a non-NLR family member belonging to the interferon-inducible HIN-200 protein family, was identified as a cytosolic double-stranded DNA (dsDNA) sensor facilitating formation of the AIM2 inflammasome. The AIM2 PYD interacts with adaptor protein apoptosis-associated speck-like protein (ASC) to recruit caspase-1 via its CARD and results in caspase-1-dependent IL-1β induction [143-146].

The initial description of the inflammasome was established on observations showing that formation of the human NLRP1 inflammasome was required for activation of the pro-inflammatory protease, caspase-1[137, 147]. MDP has been identified as inducer for the NLRP1 inflammasome and IL-1β secretion, in which NOD2/NLRP1 complexes are believed to mediate the process of caspase-1-dependent-IL-1β secretion [148], although no evident MDP-NLRP1-interacting has been found [149]. In addition, MDP was found to induce the activation of NLRP3 inflammasome [22, 150]. The activation mechanism of the NLRP3 inflammasome requires induction of NLRP3 expression mediated by NF-κB [150-152]. A recent study revealed that NLRP3 variants contribute to the CD susceptibility, indicating an important interference of the inflammasome in the pathogenesis of CD [153]. The gain-of-function mutation in NLRP3 leads to elevated levels of IL-1β produced by human monocytes THP-1 cells [154]. This finding might also be associated with other severe inflammatory disorders, e.g. Muckle-Wells syndrome, familial cold autoinflammatory syndrome and neonatal-onset multisystem inflammatory [155, 156].

Other MDP-dependent pathways and clinical implications

Decreased bone mineral density (BMD) is a common extraintestinal complication in IBD [157-159]. The balance between bone resorption and formation, i.e. the process of bone remodeling, is tightly regulated by osteoclasts and osteoblasts, respectively [160]. In recent studies, MDP has been found to contribute synergistically to the enhancement of osteoclasts formation through elevated NOD2-dependent expression of RANKL osteoblasts [161]. These observations suggest that MDP might play a key role in osteoclastic bone resorption in inflammatory bone diseases.

Moreover, MDP can elicit divergent biological effects dependent on the activated biological switch. For instance a number of apoptotic pathways are induced by the MDP-dependent pathway, such as the calreticulin (CRT) and the ASC-mediated pathway [162, 163]. MDP activation of CRT, an endoplasmic reticulum Ca^{2+} binding chaperone with multiple functions [164], results in the induction of a conformational change of TNFR1-associated death domain protein (TRADD) or Fas associated death domain protein (FADD), which subsequently activates caspase-8 and thereby cell death in cell lines such as rabbit kidney epithelial cells and Hela cells [163, 165]. Normalization of this apoptotic pathway might imply a promising treatment procedure for IBD. For instance repressing of FADD in mouse model of acute experimental colitis leads to a modulation of TNF-mediated intestinal epithelial apoptosis [166].

In addition to its role as an adaptor protein linking NLR proteins with caspases, the ASC protein interacts with MDP and activates caspase-8-mediated apoptosis [162]. Previously, ASC has been shown to be involved in the chemosensitivity of cancer cells [167], and the ASC-mediated apoptosis might thus be a potential target in oncology.

Concluding remarks

In combination with genetic and environmental factors, the luminal bacterial flora plays a pivotal role in the initiation and perpetuation of IBD. An analysis of published research shows that MDP induces a wide range of biological effects, and further, that impairment of MDP related pathways might contribute to the inflammatory process in CD. Thus, MDP can activate divergent extra- and intracellular PRRs and utilize several strategies for intracellular delivery. NOD2 is a well-known innate cytosolic receptor that senses the intracellular MDP. MDP-NOD2 interaction has been shown to activate NF-κB-dependent pro-inflammatory and antibacterial responses. Mutations in *NOD2* are associated with an early onset and a more complicated clinical course of CD including fibrostenosis and fistulization, but the functional

link between these mutations and CD has not been entirely elucidated. There are several possible mechanisms for a dysfunctional role of NOD2 in CD susceptibility. It has been suggested that defects in NOD2 signaling polarize the adoptive immune towards Th1/Th17-type, which leads to an excessive Th1/Th17 cell-mediated inflammation. In addition, NOD2 signaling has been reported as both a negative and a positive regulator of TLR responses. A *NOD2* mutation can drive Th1 immune response by negative regulation of TLR2, whereas NOD2 can also provide a synergistic effect with TLR9 in maintaining intestinal homeostasis. An impaired NOD2 interaction with ATG16L1, and thereby impaired autophagy causing defect bacterial handling, is another likely mechanism of CD associated dysbiosis.

Recent studies have expanded the induction ability of MDP beyond interaction with NOD2. Indeed, MDP has been shown to interact with inflammasomes, through NLRP1 and NLRP3, and to induce production of the pro-inflammatory cytokines belonging to the IL-1 family of cytokines. Further, NOD2 signaling might be impaired even in CD patients with the *wild*-type *NOD2* gene variant, suggesting that epigenetic changes and alternations downstream to NOD2 could play a key role in the bacteria host interaction in CD [168]. The understanding of how interaction between MDP producing bacteria and the innate immune system contributes to the pathogenesis of CD therefore relies on a better understanding of how shutting down of one part of the MDP response (*e.g.* by defect NOD2 signaling), poses imbalances towards other parts of the NOD2 response (*e.g.* non-NOD2 dependent signaling). Forcing or enhancing signaling through these pathways by stimulation with small molecules could in this way downgrade the imbalance and normalize the innate immune response, and thereby reduce the inflammatory burden in CD, as it has been observed in experimental colitis [60, 169].

In this review several findings related to the molecular mechanisms of intracellular delivery of MDP and its biological effect in the CD pathogenesis have been highlighted. Furthermore, the cross-talk between NOD2 and other innate signaling pathways of relevance to disease pathogenesis have been described, as well as the role of MDP in the inflammasome-mediated and various other pathways (e.g. NOD2 and TLR2). Altogether, these observations raise questions on the responsive roles of MDP for the innate immunity and CD pathogenesis, and it is assumed that clarification of the impact of MDP could possess major implications of importance for future treatment strategies for CD.

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Legends to figures

Figure 1. Mechanisms of MDP intracellular delivery. (A) Extracellular MDP can permeabilize the host cell through membrane receptors such as hPepT1 and Pennexin-1. (B) Extracellular MDP can also be transported into the host cell by clathrin- and dynamin-dependent endocytic pathways. (C) Moreover, MDP can be derived from bacteria by the intracellular phagocytic cleavage. (D) Finally, MDP can additionally be released to the cytosol through autolysis of internalized of invasive bacteria.

Figure 2. Structure of NOD2. The structure of NOD2 consists of two N-terminal caspase recruitment domains (CARDs) which mediate protein-protein interactions, a centrally located nucleotide-binding domain (NBD) which mediates protein self-oligomerization required for activation, and C-terminal of site of ligand binding, i.e. leucine-rich repeats (LRRs).

Figure 3. Signaling pathway of NOD2. Recognition of MDP through LRRs domain activates NOD2 and causes a conformational change, allowing a CARD-CARD interaction between NOD2 and RIP2; receptor-interacting protein 2. The activation of RIP2 upon binding with NOD2 results in polyubiquitination (ub) of NEMO; NF-κB essential modifier (or IKKγ; inhibitor of IκB kinase γ), which is found in complex with IKKα and IKKβ. This is followed by the phosphorylation of IKKβ as well as the phosphorylation of IκB; inhibitor of NF-κB, and the release of NF-κB. NF-κB can subsequently translocate into the nucleus and promote transcription of many pro-inflammatory and anti-inflammatory cytokines, including interleukin-1 (IL-1), IL-2, IL-6, IL-8, IL-12, and TNF-α.

Figure 4. TLR2 activation with MDP. Binding of MDP to TLR2 starts a signaling cascade initiated by activation of MyD88; myeloid differentiation primary-response protein 88. This cascade activates tumor-necrosis-factor-receptor-associated factor 6; TRAF6, or by binding to receptor-interacting serine/threonine kinase; RICK, resulting in induction of MAPK- and NF-κB-mediated responses, such as inflammation and Th1/Th2 activation.

Figure 5. MDP activation of pro-inflammatory cytokines through inflammasome-mediated pathway. The prototype structures of NLRP1, NLRP3 and NLRC4 consist of the following major interaction domains: The ligand sensing leucine - rich repeats (LRR), NACHT or the oligomerization domain, pyrin domain (PYD) or caspase recruitment domain (CARD), and function to find domain (FIIND). MDP interaction with these NLRs leads to the formation of inflammasomes, which are able to convert pro-inflammatory cytokines into their biologically active form.